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Thirteenth day of September 2000

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TREATMENT OF ACINAR CELL PANCREATIC CARCINOMA

Field of Invention

The present invention relates to treatment of pancreatic diseases of humans and animals. This invention has particular but not exclusive application for treatment of acinar cell carcinoma, mixed cell (including acinar cell) pancreatic carcinoma, acute and chronic pancreatitis.

Prior Art

Cyanohydroxybutene (CHB) is glycosinolate breakdown product found in cruciferous vegetables, raw canola, and many stock feeds. It has been observed that cyanohydroxybutene (CHB) administered by gavage with a daily dose of 200 mg/kg to rats for four days caused acinar cell apoptosis, inflammation, and exocrine pancreatic atophy (Wallig et al. 1998, Fd Chem Toxic 26:137-147).

Histological and ultrastructural evaluations were conducted on rats at different time periods after administration by gavage of 200 mg CHB/kg body weight in corn oil. These investigations revealed that as early as 4 hours after CHB administration, the pancreas exhibited abnormal pathology including mild to moderate supranuclear vacuolation of acinar cells. After 24 hours of CHB administration, the rats exhibited acinar cell apoptosis with cytoplasmic basophilia, lack of zymogen, diffuse vacuolation, clumping of chromatin, and nuclear pyknosis or karyorrhexis (Wallig and Jeffery 1990, Fund. Appl. Toxicol. 14:144-159).

Synthetic CHB being racemic mixture of the R- and S- enantiomers administered by gavage in olive oil at doses of 25-200 mg/kg body weight causes similar effects in the pancreas of rats as naturally occurring CHB. It was observed

that pancreatic edema and acinar cell vacuolation and depletion of zymogen granules occurred within hours of administration (Maher et al. 1991, Pancreas 6:168-175).

A single dose of 50 mg CHB/kg was administered intravenously to rats and found to form apoptotic bodies in the pancreas whereas a single dose of 100mg CHB/kg was found to cause severe pancretotoxicity with necrosis (Wallig et al. 1992 Fundamental Applied Toxicology 19:598-606).

The pancreas is a secretory gland comprising approximately 80% of acinar cells, 1% to 2% of islet cells in clusters, and 10% to 15% of single layered cuboidal ductal cells interlaced with blood vessels, lymphatics, nerves, and collagenous stroma (Evans et al. Cancer of the Pancreas. In Cancer: Principles and Practice of Oncology, 5th edition, DeVita et al. (Eds), Lippincott-Raven, New York).

Despite the large population of acinar cells, acinar cell carcinoma only accounts for 1% - 3% of pancreatic carcinomas. In addition only 5% - 10% of pancreatic carcinomas comprise mixed cell populations including acinar cells (Nonomura et al., 1992 Ultrastructural Pathology 16:317-329; Cubilla and Fitzgerald 1975 Cancer Research 35:2234-2248). Pancreatic carcinomas and acinar cell carcinomas have been reported to be aggressive diseases with a high fatality rate (Klimstra et al., 1992, Am. J. Surg. Pathol. 16(9):815-837; Adis Editors. 1997, The Oncology Review 2-4). Surgical resection is the recommended treatment for pancreatic carcinomas. However even with reductions in operative mortality following surgical resection, the survival rate from pancreatic carcinoma has changed little from its initial description in 1935 with the current 5 year survival rate being between 2% and 5% (Adis Editors. 1997, The Oncology Review 2-4).

Another disease of the pancreas is acute pancreatitis which appears to have variable severity. Acute pancreatitis appears to arise when the pancreatic duct is

obstructed by a gallstone or tumour, or when toxins to the pancreas such as ethanol are ingested. Enzyme production continues causing digestion of the pancreas with often fatal results. Recurrent bouts of acute pancreatitis (or chronic pancreatitis) often result in scarring and deformation of the ductal system thereby causing localised obstruction and thus perpetuating pain, disability and digestive deficiency. The events which regulate the severity of acute pancreatitis are unknown. Several studies, however, have shown that mild pancreatitis was found to be associated with extensive apoptotic acinar cell death while severe pancreatitis was noted to involve extensive acinar cell necrosis but very little acinar cell apoptosis (Kaiser et al. 1995, Am. J. Physiol. 269:C1295-C1304: Gukovskaya et al. 1996 Gastroenterology 110:875-884). In a further study the relationship between acinar cell apoptosis and the severity of pancreatitis was investigated by administering a single intravenous dose of CHB (70mg/kg) in corn oil to mice and inducing pancreatitis at varying times after CHB administration. They found that the severity of pancreatitis is reduced when the disease is induced during the period in which morphologic evidence of apoptosis is most extensive but induction of pancreatitis either before or after the extent of apoptosis has peaked results in pancreatic injury which is similar to that noted in animals not exposed to CHB (Bhatia et al.1998, Biochem. Biophys. Res. Commun. 246:476-483). It appears that the acinar cells regenerated after treatment with CHB. The practical usefulness of the method to treat acute pancreatitis therefore appears limited.

Summary of the Invention

It is an object of the present invention to provide a method of treating acinar cell carcinoma and some mixed cell carcinomas that include acinar cells with administration of CHB.

The present invention arises from the surprising discovery that subcutaneous injection of CHB produces an unusual and unsuspected result of acinar cell apoptosis of normal acinar cells and acinar cell carcinoma. It was found that subcutaneous injection of CHB at an appropriate sub-lethal dosage caused apoptosis of the substantially entire population of acinar cells. The pancreatic lesion was unusual in that there was observed a marked early edema with limited inflammatory infiltration, rapid synchronous onset of acinar cell apoptosis and advanced atrophy engendering only a severely limited regenerative response. The application of this discovery to treat acinar cell carcinoma has lead to the development of the current invention.

The present invention in one aspect broadly resides in a method for treating acinar cell carcinoma including preparing a cyanohydroxybutene (CHB) formulation; and administering a one or more sub-lethal doses of the CHB formulation to a subject with acinar cell carcinoma.

The CHB formulation is preferably a CHB solution wherein CHB is substantially dissolved in water. The CHB may be a natural or synthetically derived CHB. The CHB dose is preferably calculated within a range of 5-300mg CHB/kg of body weight. More preferably the CHB dose is 150 mg/ kg of body weight. The dosage may vary between subjects. Subjects include animals and people of different sizes and weight. The dose is preferably of a concentration that causes apoptosis of substantially all acinar cells and substantially no regeneration of the acinar cells for an extended period of time.

Administration is preferably by means where the CHB is absorbed slowly and substantially avoids the liver. Preferably the CHB dose is administered by subcutaneous injection. In an alternative form the CHB dose is administered so that it is delivered directly to the acinar cells. In this form the CHB molecule may be conjugated to a ligand molecule which is able to bind to an acinar cell surface receptor thereby delivering CHB to the acinar cell.

Acinar cell carcinoma includes carcinomas of only acinar cells or of mixed cell populations with a proportion being acinar cells.

The above method of treating acinar cells may be applied to the treatment of acute and chronic pancreatitis. Thus in another aspect the present invention broadly resides in a method for treating acute or chronic pancreatitis including preparing a cyanohydroxybutene (CHB) formulation; and administering one or more sub-lethal doses of the CHB formulation to a subject with acute or chronic pancreatitis wherein the dose of CHB has a concentration that causes apoptosis of substantially all acinar cells and substantially no regeneration of acinar cells for an extended period of time

The description of the features of the method for treating acinar cell carcinoma apply also to the above method where applicable.

Brief description of the Drawings

In order that the invention may be more readily understood reference will now be made to the accompanying drawings which illustrate the experimental results and a preferred embodiment of the invention and wherein:

Figure 1 shows body weights of animals as a percentage of starting weight after a single subcutaneous injection of saline or CHB (n=4, results expressed as means ± SEM.);

Figure 2 shows pancreatic weight as a percentage of body weight in animals after a single subcutaneous injection of saline or CHB (n=4, results expressed as means ± SEM.);

Figure 3 shows pancreatic morphology after a single subcutaneous injection of saline of CHB. All H&E. **(A)** 48 hours after saline. There is wide separation of ducts (arrows) and islets (I) by closely-packed acinar cells (x500). **(B** 12 hours after CHB. Note numerous apoptotic acinar cells with characteristic nuclear morphology (arrow) (x1200). **(C)** 18 hours after CHB. Most acinar cells contain pyknotic nuclear remnants (arrowheads) and show cytoplasmic swelling and vacuolation; a few appear normal (arrows) (x900). **(D)** 48 hours after CHB. Advanced secondary necrosis affecting all acinar cells in field. Intact duct is indicated by arrow (x360). **(E)** 96 hours after CHB. No acinar cells remain. Atrophic lobules comprise crowded ducts in a connective tissue stroma. (x200). **(F)** 28 days after CHB. Sparse regenerative acini are seen adjacent to islets (arrows). Note few ducts in a collagenous stroma and prominent fatty infiltration, (x200);

Figure 4 shows pancreatic immunohistochemistry after a single subcutaneous injection of saline or CHB. All with haematoxylin counterstain. **(A)** 24 hours after saline. Widely-spaced keratin-positive ducts (arrows) are separated by closely-packed keratin negative acinar cells (x160) **(B)** 48 hours after CHB. Widely-spaced keratin-positive ducts (arrows) separated by keratin-negative nonviable acinar cells (x180). **(C)** 96 hours after CHB. Lobules comprise crowded keratin-positive ducts separated by loose connective tissue. No acinar cells are seen, (x180). **(D)** 96 hours after CHB. Ducts are negative for amylase. Note isolated amylase positive epithelial cell (arrow) and perinsular amylase positivity (x160);

Figure 5 shows pancreatic ultrastructure after a single subcutaneous injection of CHB. **(A)** 12 hours after CHB. Adjacent apoptotic acinar cells show well-demarcated crescentic clumped chromatin, large nucleolar remnants (Arrowhead) and whorling of endoplasmic reticulin (arrows) (x3000). **(B)** 18 hours after CHB. Apoptotic acinar cells show nuclear fragments with crescentic clumped chromatin but dilation of endoplasmic reticulin, swollen mitochondria (arrowheads) and plasma membrane rupture (arrow). Contrast with adjacent viable acinar cells (x2800). **(C)** 48 hours after CHB. Note viable duct epithelial cells (D), residual acinar cell cytoplasmic debris (A) and intraacinar macrophage (M) laden with residual bodies. The pale cell in the duct epithelium (arrow) is also likely to represent and intraepithelial macrophage (x350). **(D)** 96 hours after CHB. Duct with typical indented nuclei and sparse organelles of lining cells. Note mitotic lining cell (M), intraepithelial apoptotic body (arrow), and adjacent collapsed redundant basement membrane (arrowhead) (x2500). **(E)** x hours after CHB. Activated and mitotic interstitial fibroblasts. (x.3300). **(F)** 48 hours after CHB. Capillary with intraluminal apoptotic bodies of presumed endothelial cell origin (arrow). Note adjacent intraacinar macrophage (M) with residual body-laden cytoplasm (x.7000);

Figure 6. **(A)**. Untreated AR42J acinar cell carcinoma in an athymic rat, showing broad sheets of cells with an area of haemorrhagic necrosis (arrow) (x100). **(B)** Untreated AR42J acinar cell carcinoma, (x400), showing multiple mitoses. **(C)** AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing widespread apoptosis and an area of surviving cells (arrow). (x100). **(D)** AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing widespread apoptotic change (x400).

Figure 7 **(A)** Untreated AR42J acinar cell carcinoma, showing primitive acini formation (x1000). **(B)** AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing pyknotic and fragmented nuclei and secondary necrosis of cytoplasm (x1000). **(C)** Pancreas of a normal Wistar rat 24 hours after 140mg/kg subcutaneous CHB showing almost total acinar cell death via apoptosis with secondary necrosis (x400). **(D)** Pancreas of athymic rat 24 hours after 140mg/kg subcutaneous CHB showing regional effect with necrotic areas adjacent to well-preserved areas with pyknotic nuclei (x400).

Fig.8 Electronmicrographies of apoptotic cells showing condensed marginated chromatin.

Experimental

1. **Determination of Acinar Cell Apoptosis and Regeneration of Acinar Cells with Subcutaneous Administration of CHB.**

1.1 Material and Methods

Synthetic CHB made according to the method of Das and Torssell (Das and Torssell 1983 Tetrahedron 39:2243-2247).

Male Wistar rats weighing 200 – 250 g were caged in pairs and given food and water ad libitum with a 12 hours light-dark cycle. Twelve groups of 10 rats were divided randomly into 6 test animals and 4 control animals. At time 0, test animals were given 150mg/kg of CHB mixed in 0.5ml sterile normal saline and controls were given 0.5ml sterile normal saline subcutaneously.

For light microscopy, 4 experimental and 4 control animals were killed at 2, 4, 6, 12, 24, 48, 72 and 96 hours and 7, 10, 18 and 28 days using 60 mg intraperitoneal

pentobarbitone. Animals were weighed and the pancreas removed, weighed and processed using routine methods. Additional pairs of experimental animals were killed at 18 and 60 hours for electron microscopy and morphological study. Weights were recorded as means ± standard error of the mean (SEM). Differences between means were analysed using Student's t-test.

For quantification of apoptosis, apoptotic cells and bodies, identified using the morphological criteria (Kerr et al. 1995 Method Cell Biol. 46:1-27) and were counted in ten high-power fields (HPF), selected at random, in a histological slide from each animal at 2, 4, 6 and 12 hours with the proviso that mostly acinar tissue filled the field. A group of tightly clustered apoptotic bodies, presumably derived from a single cell, was recorded as a single count. An estimate of the total number of acinar cells per HPF in each slide was made for calculation of an apoptotic index (apoptotic count as a percentage of total acinar cells present). Counts/HPF and apoptotic indices were recorded as means ± SEM for each group. Differences between means were analysed using Student's t-test. Terminal d-UTP nick-end labelling (TUNEL) was not used because it is our experience and the experience of others that it is not always specified for apoptosis, and ultimately, apoptosis must be confirmed morphologically (Ansari et al. 1993 J. Pathol. 170:1-8; Grasl-Kraupp et al. 1995 Hepatology 21:1465-1468).

Immunohistochemistry for cytokeratin an amylase was performed to identify cells in sections as duct (Schussler et al. 1992 Am. J. Pathol 140:559-568; Bouwens et al. 1995 J Histochem Cytochem. 43:245-253) or acinar (Bendayan 1984 Histochem J. 16:85-108) respectively. For cytokeratin, deparaffinized sections were pretreated with 0.1% trypsin, then 0.3% hydrogen peroxide in methanol followed by mouse monoclonal AE1/AE3 anti-cytokeratin at a dilution of 1/40. Secondary

antibody was rat anti-mouse biotinylated IgG used at a dilution of 1/400. Antibody-binding was demonstrated using the peroxidase-labelled streptavidin biotin complex method and reactions were developed with 3,3'-diaminobenzidine tetrahydrochloride solution. For amylase, deparaffinized sections were boiled in Target Retrieval Solution then placed in 0.3% hydrogen peroxide in methanol. Primary antibody was anti-rabbit immunoglobulin used at a dilution of 1/500 and secondary antibody was anti-rabbit goat biotinylated IgG used at a dilution of 1/400. Antigen-binding was demonstrated using the peroxidase-streptavidin method developed with Vector VIP peroxidase substrate. All sections were lightly counterstained with hematoxylin.

For electron microscopy, two rats from each test group were deeply anaesthetised with intraperitoneal sodium pentobarbitone. A catheter was retrogradely inserted into the abdominal aorta and the vasculature flushed in sequence with 1) heparinized normal saline, 2) 1% paraformaldehyde and 1.2% glutaraldehyde in cacodylate buffer and 3) 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (Karnovsky 1965 J, Cell Biol.27:137A-138A). Pancreas was removed immediately, diced and immersed in perfusate no. 3 for two hours, then stored in cacodylate buffer. The tissue was postfixed in 1% osmium tetroxide, stained en bloc in 5% aqueous uranyl acetate, dehydrated through a series of graded alcohols, cleared in propylene oxide, and embedded in an epon-araldite mixture. Semithin sections (1μm) were cut on an LKB Ultratome V and stained with toluidine blue for viewing. Ultrathin sections from selected areas were picked up on uncoated copper grids, stained with lead citrate and examined with a JEOL-1200 EX11 electron microscope.

1.2 Results

1.2.1 General Observations

Control rats showed no behavioural change and normal weight gain reaching 180% at 28-days (Figure 1). Experimental rats showed discomfort 30 minutes after injection, lost curiosity and became reluctant to move. Body weight fell over the first week and thereafter remained unchanged (Figure 1).

At autopsy, control rats had normal viscera and a pancreatic weight which was constant as a proportion of body weight (Figure 2). Experimental animals showed pancreatic edema from 2 hours, actual pancreatic weight reaching 4.41±0.71 g at 6 hours (compared to 0.78±0.14g in controls, $P<0.001$), then falling. Atrophy was apparent at 7 days and persisted, actual pancreatic weight falling to 0.44 ±0.04g at this time compared to 1.80±0.08g in controls, $P<0.001$.

Changes in pancreatic weight as a percentage of body weight are shown in Figure 2. At 18 and 28 days CHB-treated rats had muscle wasting, abdominal distension and dilated bowel containing undigested food.

1.2.2 Light Microscopy

Control animals showed histologically normal pancreas (Figure 3A). From 2 hours test animals showed mild dilation of acinar lumens and acinar cell vacuolation and depletion of zymogen granules.

Apoptotic acinar cells, evident at 6 hours, showed sharply-defined crescents of clumped chromatin against the nuclear envelope but infrequent fragmentation. Their number reached 178 ±10/HPF at 12 hours (compared to 0.85 ±0.13/HPF in controls, $P<0.001$) or 23.6±7.43% of acinar cells (compared to 0.001% in controls) (Figure 3

B). By 18 hours most acinar cells had chromatin changes of apoptosis but swollen vacuolated cytoplasm indicative of "secondary necrosis" (Figure 3C) which subsequently progressed (Figure 3D). By 96 hours no acinar cells remained (Figure 3E). A few regenerative acini appeared by 18 days, particularly adjacent to islets of Langerhans, but thereafter they did not increase appreciably in number (Figure 3F).

Intercalated ducts were mildly dilated at 4 hours, duct cell mitoses were prominent at 48 hours, and at 96 hours, lobules comprised groups of ducts within a connective tissue stroma (Figure 3E). Small numbers of apoptotic bodies continued to be seen within duct lumens and epithelium. By 7 days ducts had larger lumens and flattened lining epithelial cells. Thereafter the number of ducts decreased with few remaining at 18 and 28 days (Figure 3F).

Interlobular edema was present from 2 hours and interlobular edema from 4 hours; both persisted for 72 hours. The interstitial spaces were acellular before small numbers of mononuclear phagocytes appeared about vessels at 4 hours and within acini at 24 hours. They reached moderate numbers at 48 hours, peaked at 72 hours, then declined markedly by 7 days. Sparse neutrophils were present from 12 hours and mitotic mononuclear phagocytes at 48 hours.

Enlarged mitotically active fibroblasts were seen 48 hours, by 96 hours fibroblasts and collagen enveloped lobules and at 7 days fibroblasts were less prominent and collagen was found both in and around lobules. At 28 days the pancreas comprised largely fat, collagen and islets (Figure 3F).

Islets were not studied in detail. Given the degree of atrophy, however, less islet tissue was apparent than might be expected from simple condensation.

1.2.3 Immunohistochemistry

In controls ducts were positive for cytokeratin and acinar cells negative (figure 4A). At 48 hours in test animals, when few viable acinar cells remained, cytokeratin marked dispersed intact ducts and duct cells (Figure 4B). At 96 hours ducts of atrophic lobules, the only remaining epithelium, were positive for cytokeratin (Figure 4C). Amylase was demonstrated in apoptotic cells at 18 hours, confirming their acinar cell origin. There were no or rare amylase-containing cells at 72 and 96 hours (Figure 4D) with occasional apoptotic bodies staining for amylase. The periphery of islets also showed amylase staining at 96 hours (Figure 4D).

1.2.4 Electron Microscopy

Controls showed normal pancreatic ultrastructure (Ekholm et al. 1962 J. Ultrastruct. Res. 7:61-72; Ekholm et al. 1962 7:73-83). In test animals acinar cell apoptosis was slightly increased at 6 hours and markedly increased at 12 hours, when large numbers of adjacent cells were often affected (Figure 5A). Apoptotic cells showed sharply-defined crescents of chromatin abutting the nuclear envelope, prominent nuclear remnants, whorling of endoplasmic reticulum and structural preservation of organelles (figure 5A) but cellular fragmentation to form apoptotic bodies was uncommon. At 18 hours, apoptotic cells, identified by their nuclear characteristics, remained in situ, but showed dilation of endoplasmic reticulum and nuclear envelopes, swelling and rupture of mitochondria and rupture of plasma membranes (Figure 5 B), so-called "secondary necrosis". This process progressed such that, by 48 hours, acinar cells were reduced to degraded cellular material associated with small number of intraepithelial macrophages containing ingested apoptotic bodies, degraded cellular material in phagosomes of residual bodies (Figure 5C). By 96 hours, acinar cell debris had been removed (Figure 5D). Ducts

and duct cells survived, showing increased mitotic activity, particularly at 60 and 72 hours (Figures 5C and D). Small numbers of ductal intraepithelial apoptotic bodies and surrounding collapsed basement membrane were identified (Figure 5 D).

From 48 to 96 hours, prominent activated and mitotic fibroblasts were seen (Figure 5E). At first collagen was sparse but increased in amount towards 7 days. At 48 hours, mitoses in interstitial macrophages were confirmed and endothelial cell apoptosis was present in interstitial capillaries (Figure 5F); this continued over succeeding days. By 18 days, isolated regenerative acini comprised acinar cells closely resembling acinar cells in control glands.

1.3 Discussion

Within 12 hours of administration of CHB, there is relatively synchronous onset of apoptosis in the majority of acinar cells, this contrasts with the slow onset of apoptosis and gradual increase peaking about the third day that occurs after duct ligation (Walker 1987 Am. J. Pathol. 126:439-451) or the administration of cerulein (Fujimoto et al. 1997 Digestion 58:421-430) or ethionine and a protein-depleted diet (Walker et al. 1993 Pancreas 8:443-449). The sequence is similar but delayed after administration of a copper-depleted diet (Rao et al. 1993. Am. J. Path 142:1952-1957).

The rapid and synchronous onset of apoptosis after CHB administration overwhelms the capacity of duct cells, viable acinar cells and tissue macrophages to rapidly remove apoptotic cells. As a consequence, most of the apoptotic cells remain in situ undergoing progressive swelling, rupture of organelle and plasma membranes and degradative change referred to as "secondary necrosis".

A feature of the CHB model of pancreatic involution is the limited fragmentation of apoptotic acinar cells compared with that seen, for example, after duct ligation (Walker 1987 supra). In the first hours after CHB administration and at lower doses, apoptosis proceeds to cell fragmentation with intraepithelial macrophages at 12 hours engorged with phagocytosed apoptotic bodies making it unlikely that CHB prevents microfilament reorganisation.

Despite early cell death and edema, inflammatory cell infiltration is delayed, reaching moderate density only at 48 hours, the number of neutrophils remaining small throughout. In contrast, cerulein excites a vigorous inflammatory response (Walker et al. 1993 supra; Fujimoto et al 1997 supra).

Acinar cell regeneration is limited to a few acini 10-18 days after CHB administration. After cerulein and ethinione administration, it is rapid and complete once the causative agent is removed (Fitzgerald 1960 Lb. Invest. 9:67-85; Isasser et al. 1986 Pancreas 1:421-429).

2. *In Vivo* effect of CHB on Pancreatic Carcinoma

2.1 Method

Athymic rats 200 –250g were purchased from the Animal Resources Centre, Western Australia. Four experiments were performed using slightly different doses with the intention using a dose just sub-lethal in order to assess maximal effect.

Experiment 1: *Ductal* carcinoma cells (2 x 10^6 cells of Capan 2) were injected into the left flank of 7 nude rats. Sixty days later, tumour nodules were 1 –2cm diameter. By the time tumours were ready for testing, rats weighed approximately 300g. 135mg/kg CHB (absolute dose 38 - 40mg) was given into ventral abdominal

subcutaneous tissue in 0.5ml sterile saline with 2 controls getting saline only. At 18 hours, all test rats were dead. Autopsies were performed taking specimens of carcinoma, pancreas and liver for processing.

Experiment 2: 6 rats were inoculated in the right flank with 5×10^6 cells of Ar42J rat *acinar* cell carcinoma. By 13 days all grew tumours 1 – 2cm diameter. CHB at a dose of 125mg/kg mixed in 0.5ml saline (absolute dose 26 – 32.5mg) was injected into ventral abdominal skin of all 6 rats. In this experiment there were no controls. All rats survived until eighteen hours later when they were euthanased using 60mg intraperitoneal pentabarbitone. Carcinoma, pancreas and liver were dissected out and placed in formalin for processing.

Experiment 3: 14 rats were inoculated with 5×10^6 cells of Ar42J *acinar* cell carcinoma. Seven rats were given 30 – 32mg absolute or 140mg/kg and another 7 rats were given saline only. At 18 hours all rats had survived and were euthanased, pancreas, liver and carcinoma removed and taken for processing.

2.2 Results

Experiment 1: *Ductal* carcinoma nodules were not effected by CHB. Histological appearance was the same in both test and control groups.

Experiment 2: At lower doses of CHB, 1 of 6 test rats had evidence of a cytotoxic effect with widespread apoptosis and secondary necrosis. Apoptosis was as described previously, with crescentic clumping of chromatin, as well as wheel-rim clumping around the nuclear membrane. Fragmentation of cells was not obvious as

described in other settings of massive synchronous apoptosis. In order to distinguish the effect of CHB from patchy haemorrhagic necrosis, an effect was regarded as present only if little viable tumour remained.

Experiment 3: Control tumour nodules were composed of sheets of uniform large cells with dilated vascular channels and some areas of haemorrhage and necrosis (Figure 6A and B, Figure 7A). Patches of apoptotic cells were present in places, particularly near haemorrhages. Mitotic rate varied but in places was very high. Three of 7 rats had a marked effect (Figure 6C and D, Figure 7B), a further 2 had about half surviving and the remaining 2 rats had no discernible effect.

Three test rats with acinar cell carcinoma that died of CHB toxicity in preliminary dose-testing experiments had almost total cell death in the tumour nodules. The fact that one rat had some patches of surviving tumour cells makes post-mortem change unlikely to be responsible for the apoptotic appearance.

The effect on pancreas was different in athymic and Wistar rats (Figure 7C and D). Athymic rats had a regional effect in the pancreas with areas of secondary necrosis juxtaposed with well-preserved areas of acinar tissue, albeit with pyknotic nuclei.

2.3 Discussion:

There is no discernible effect of CHB on ductal cells in the normal pancreas. It is therefore not surprising that malignant ductal cells are not substantially affected by CHB. Normal acinar cells, however, are sensitive and can be eliminated by a single subcutaneous dose of 140mg/kg in the Wistar rat. Only very limited regeneration of acinar cells was seen at 28 days, and this was in the peri-islet areas.

The usual picture of apoptosis is that nuclear changes are followed rapidly by cytoplasmic condensation, blebbing and fragmentation. As described in other settings of massive synchronous apoptosis fragmentation was not obvious.

In the nude rat tumour nodules the effect of CHB appeared to be total regional apoptosis or nothing. No part effect was seen. At the lower dose, 1 of 6 rats had an almost total cell kill, 5 had no discernible effect. At the higher dose, 3 of 7 had a marked effect with few areas of viable cells, and a further 2 of 7 had apoptotic change in about half the tumour section. In unaffected tumours, there was no increase in apoptosis and no decrease in mitosis. Areas of tumour were either totally apoptotic or seemingly unaffected.

The effect on pancreas in the athymic rat is different from that in the Wistar rat. Despite marked edema as expected, the appearance of the pancreas is unusual in that nuclei in both necrotic and well-preserved areas are pyknotic. Patches of apoptotic cells are seen in the liver. It may be that a thymic humoral component is involved in the widespread apoptotic process in Wistar pancreas, without which the lesion is different.

It will of course be realised that while the foregoing has been given by way of illustrative example of this invention all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as is herein set forth.

DATED THIS THIRTIETH DAY OF AUGUST 1999.

LYNDELL KELLY

BY

PIZZEYS PATENT AND TRADE MARK ATTORNEYS

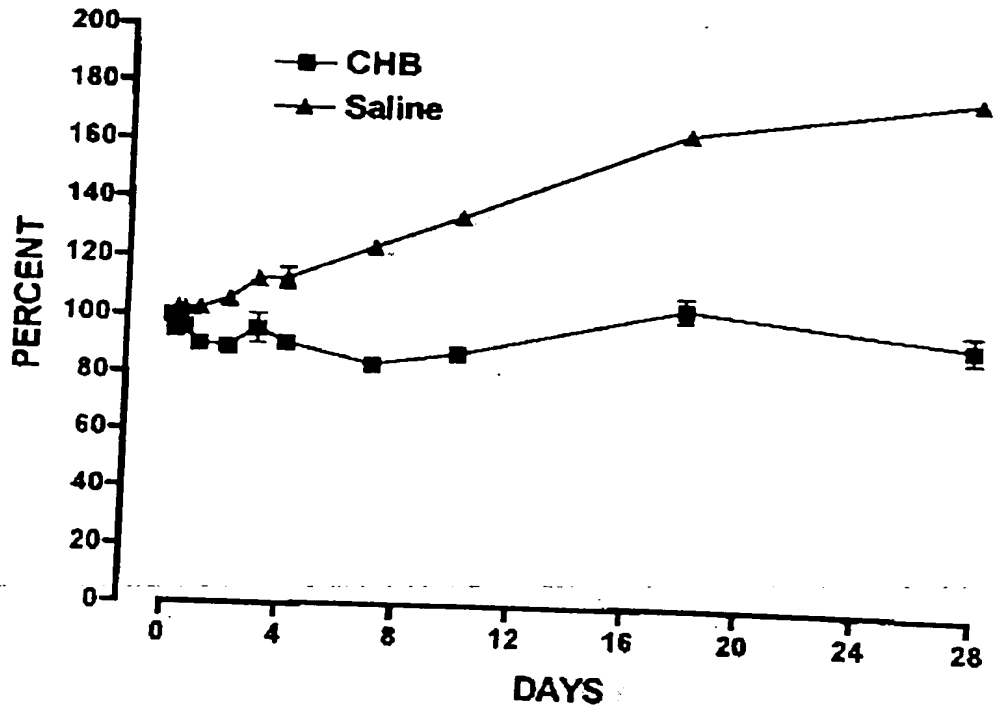
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FIGURE 1

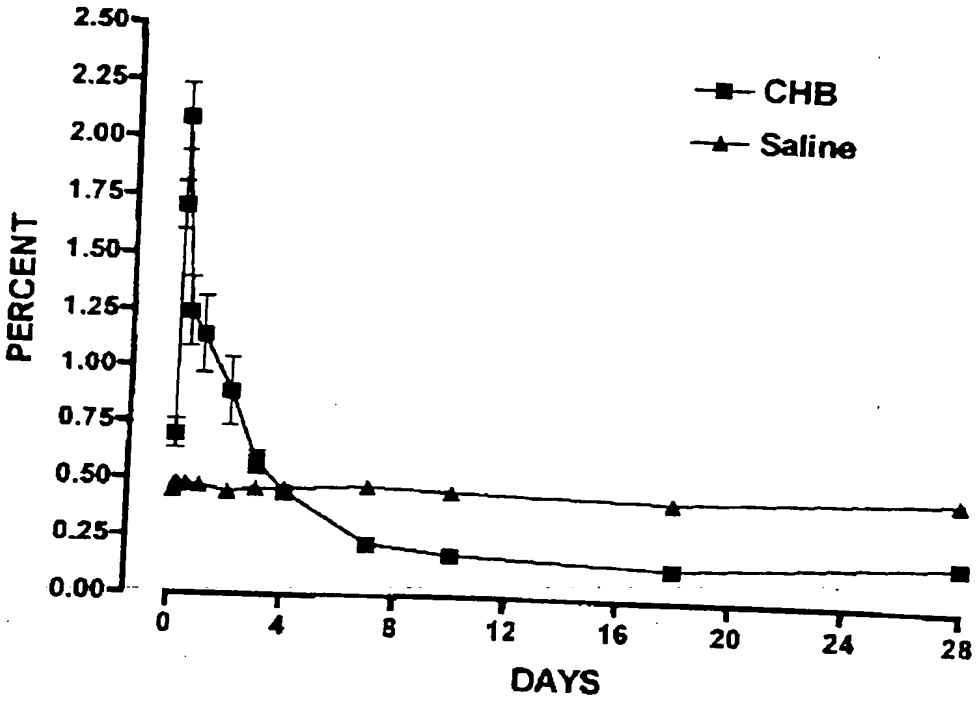
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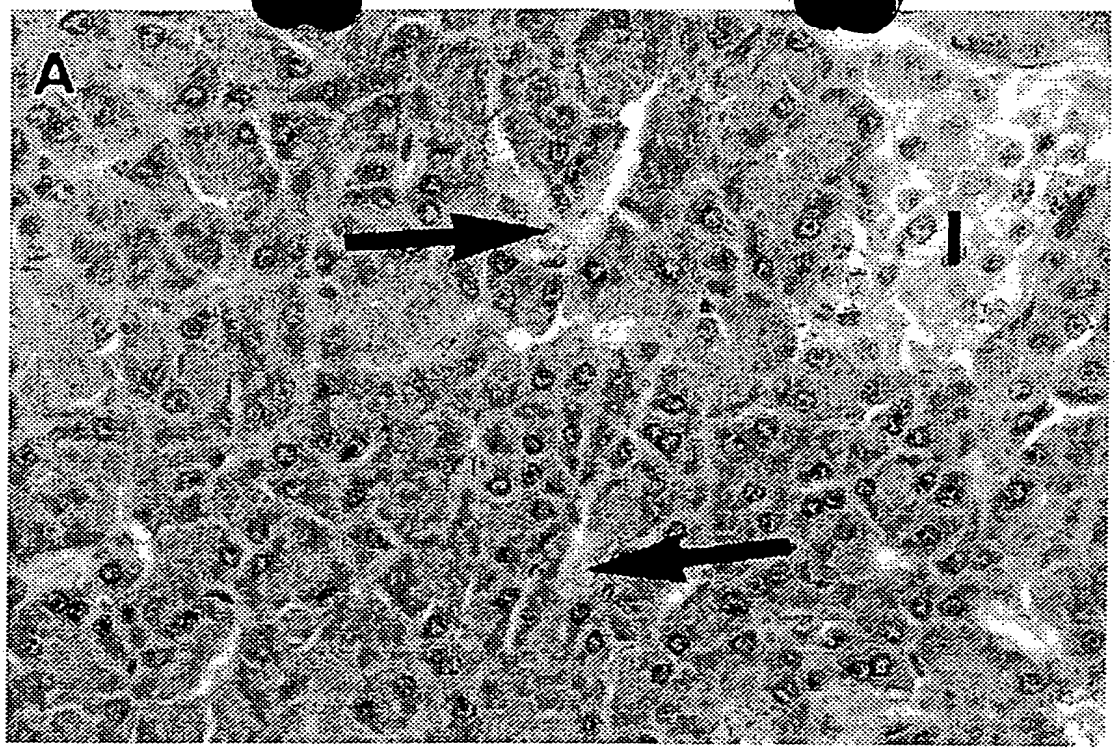
FIGURE 2



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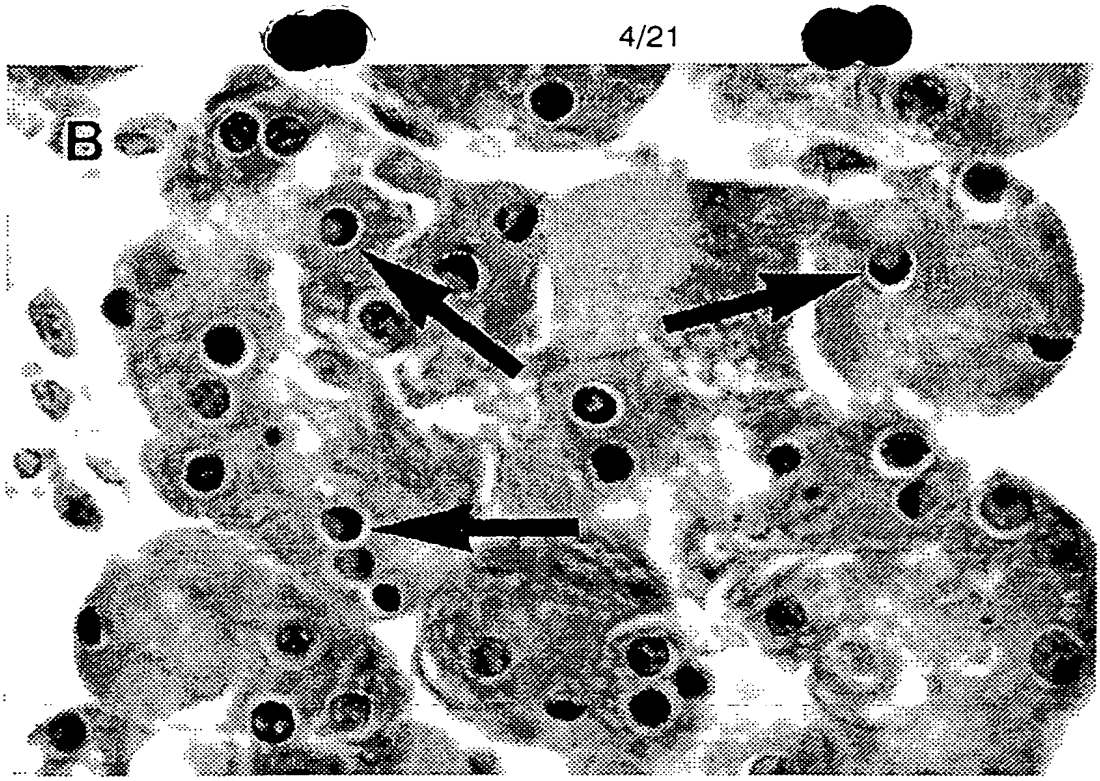

FIGURE 3

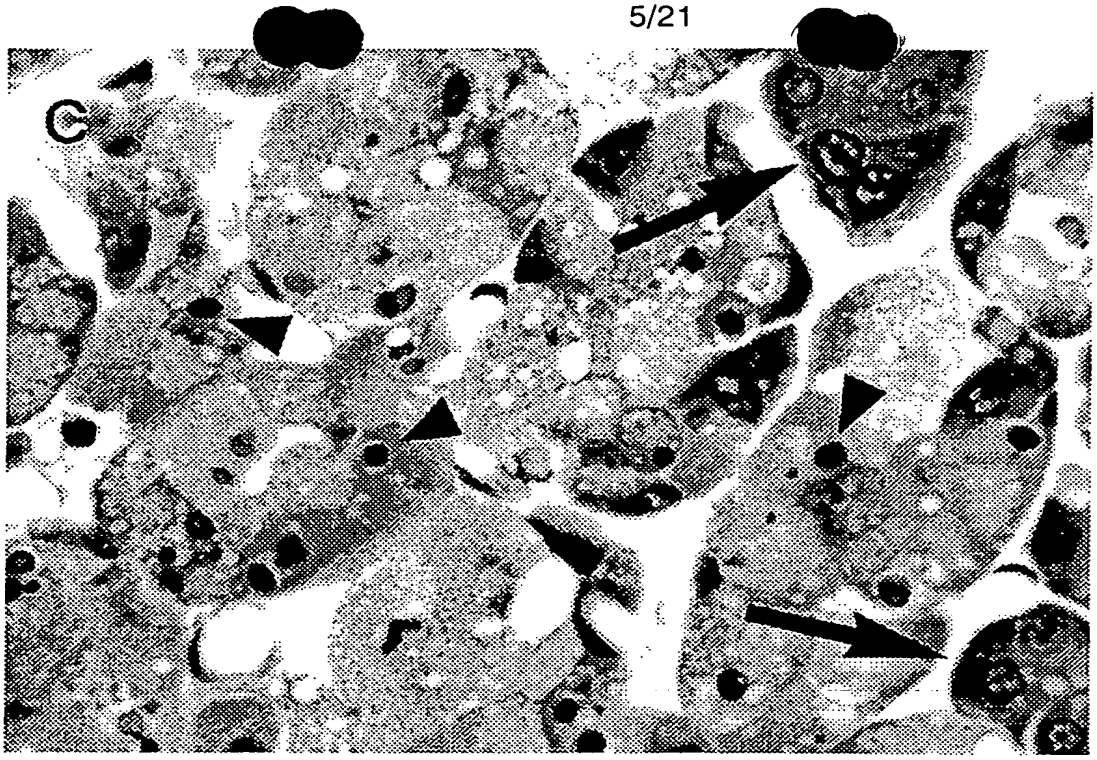

FIGURE 3

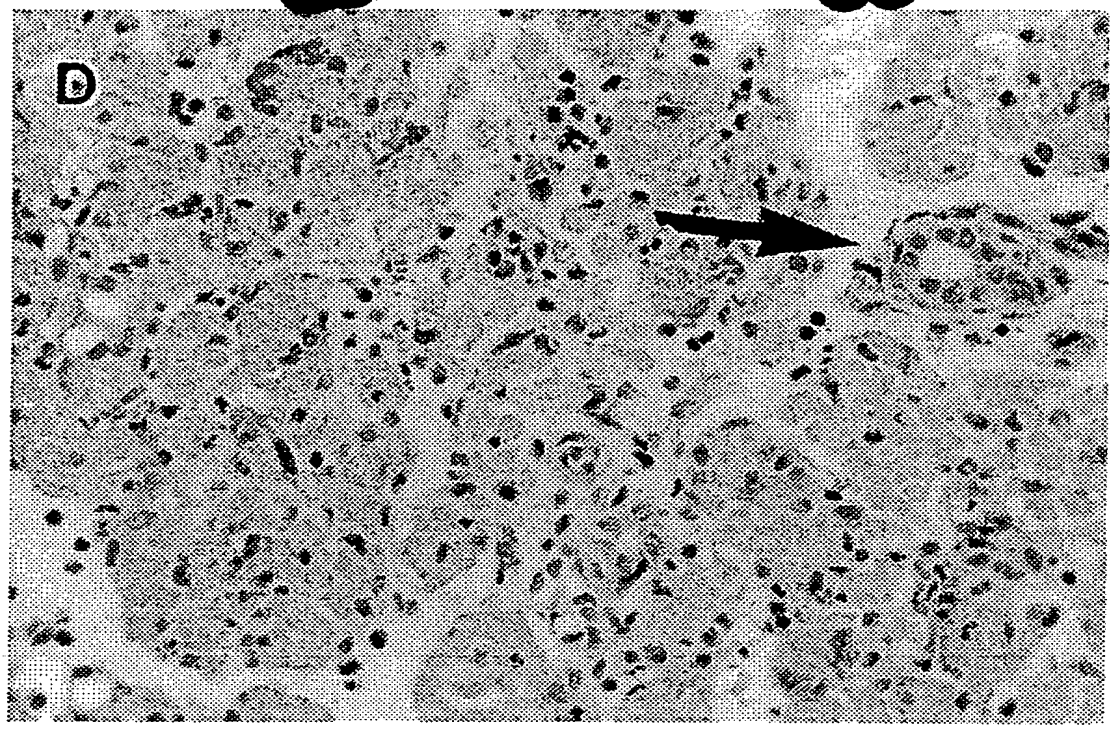

FIGURE 3

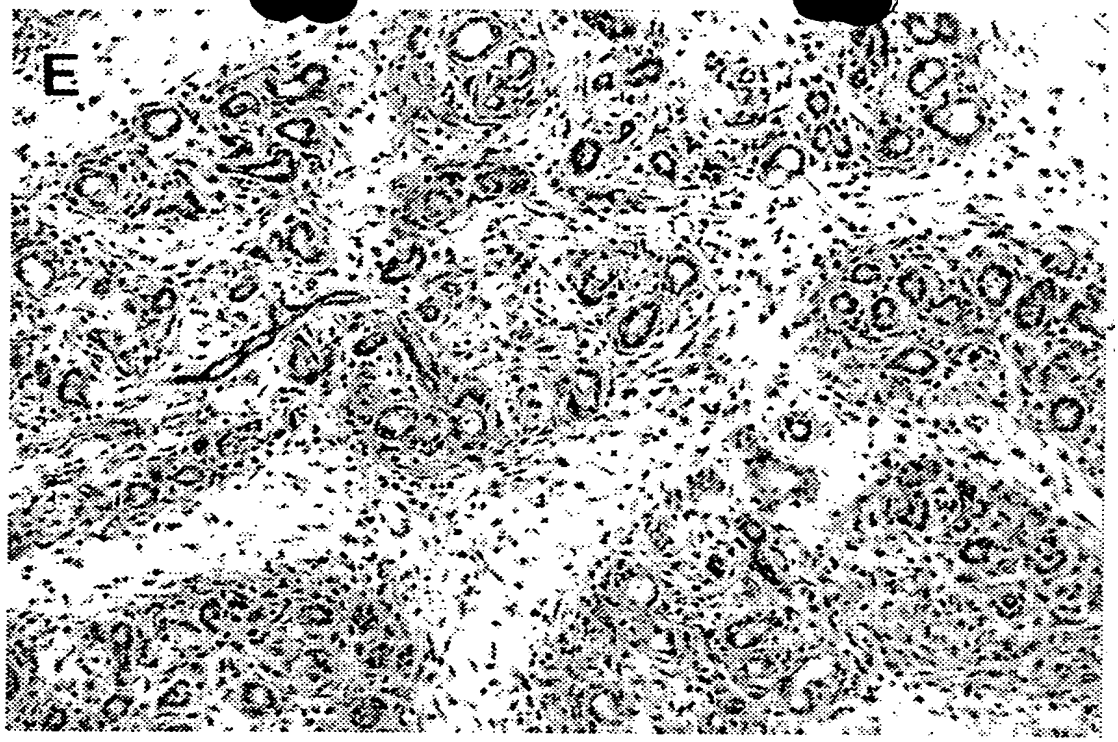

FIGURE 3

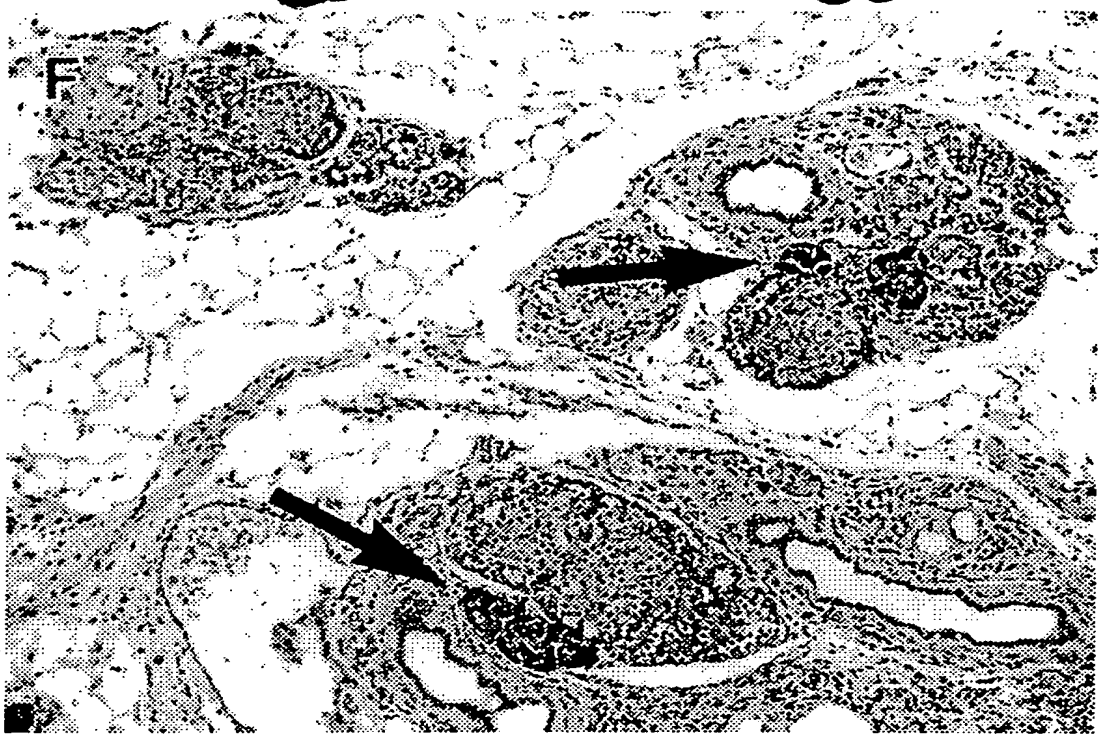

FIGURE 3

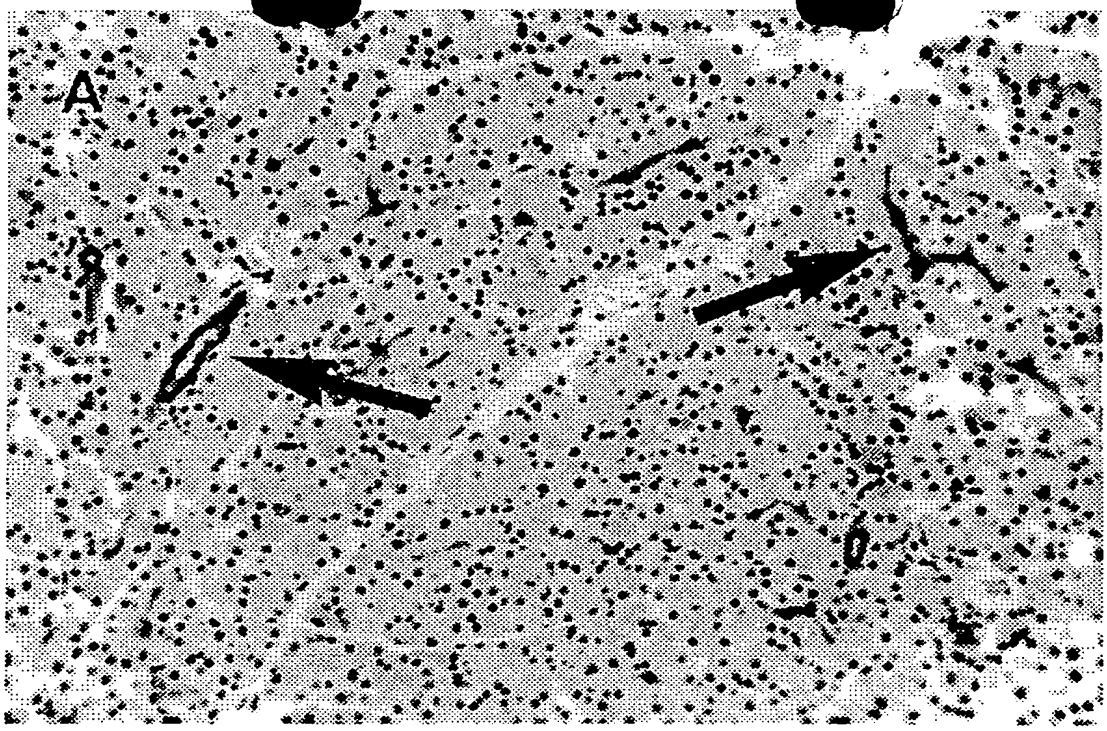

FIGURE 4

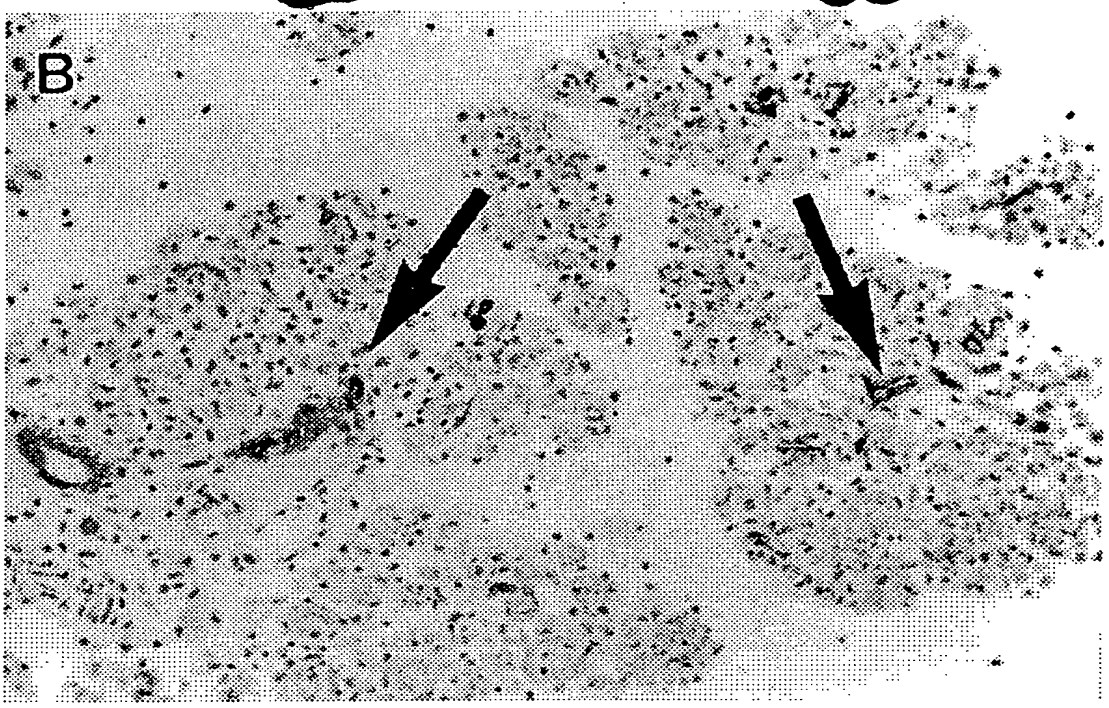

FIGURE 4

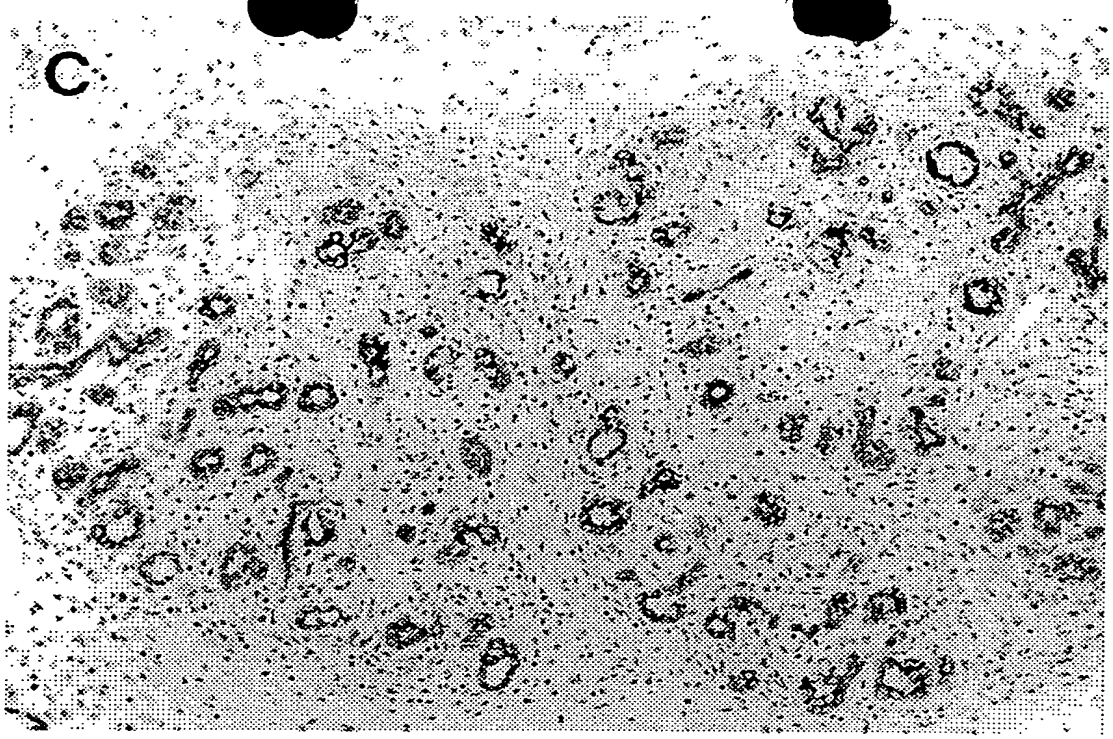

FIGURE 4

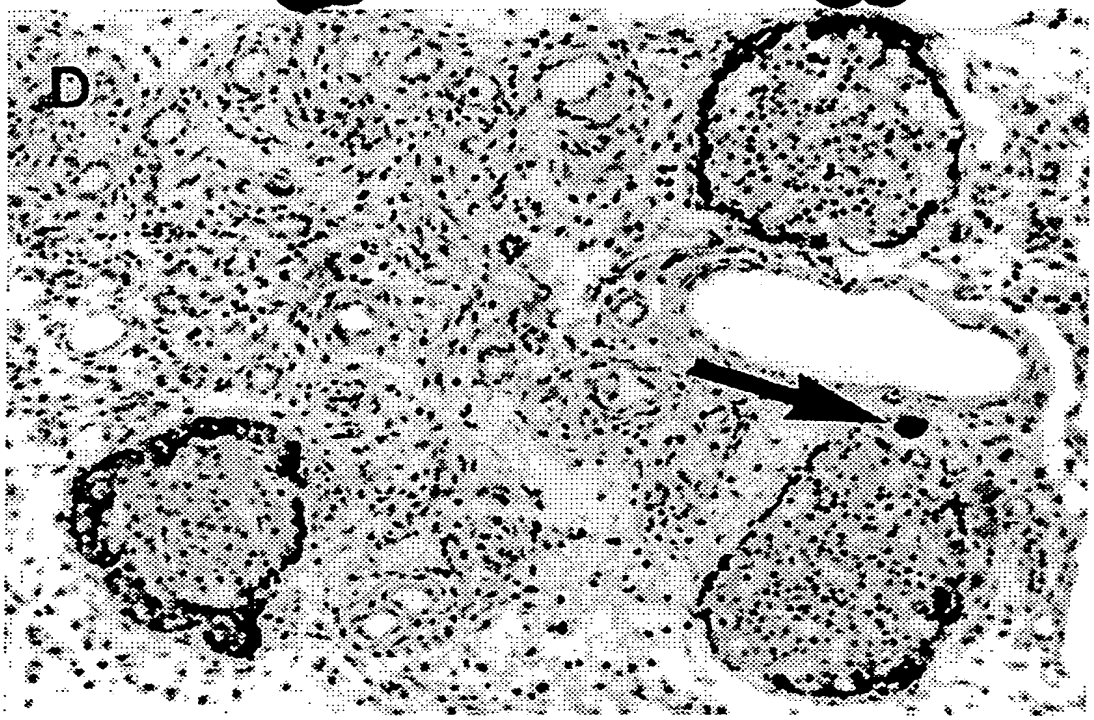

FIGURE 4

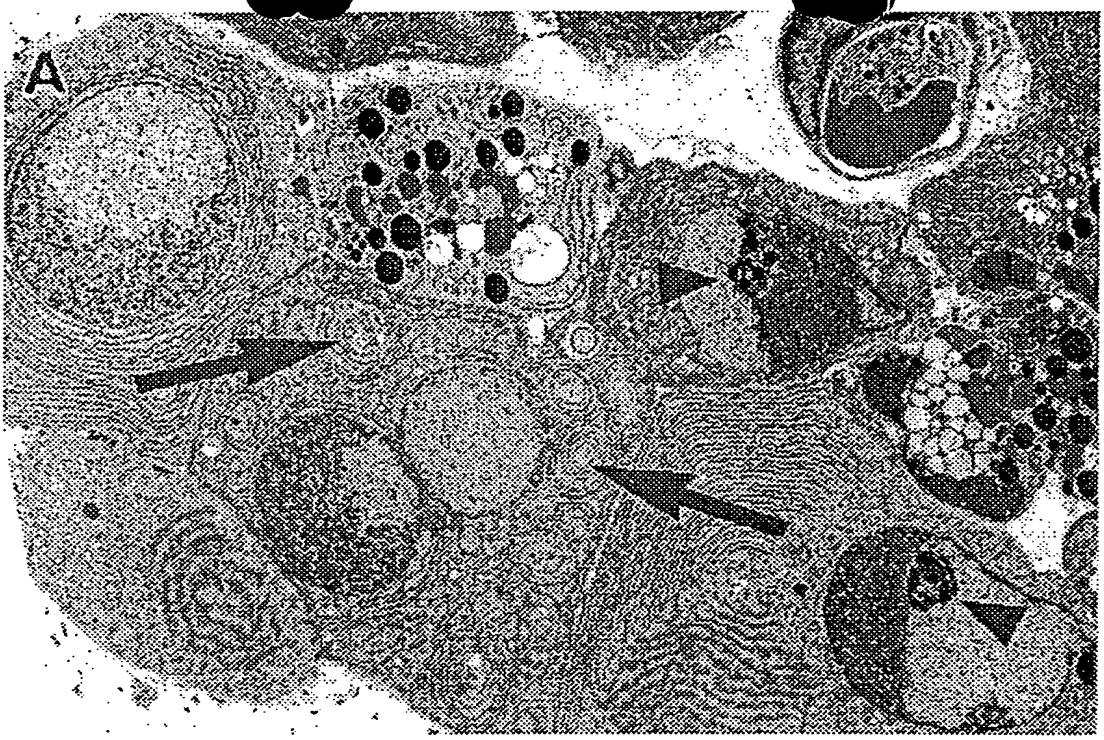

FIGURE 5

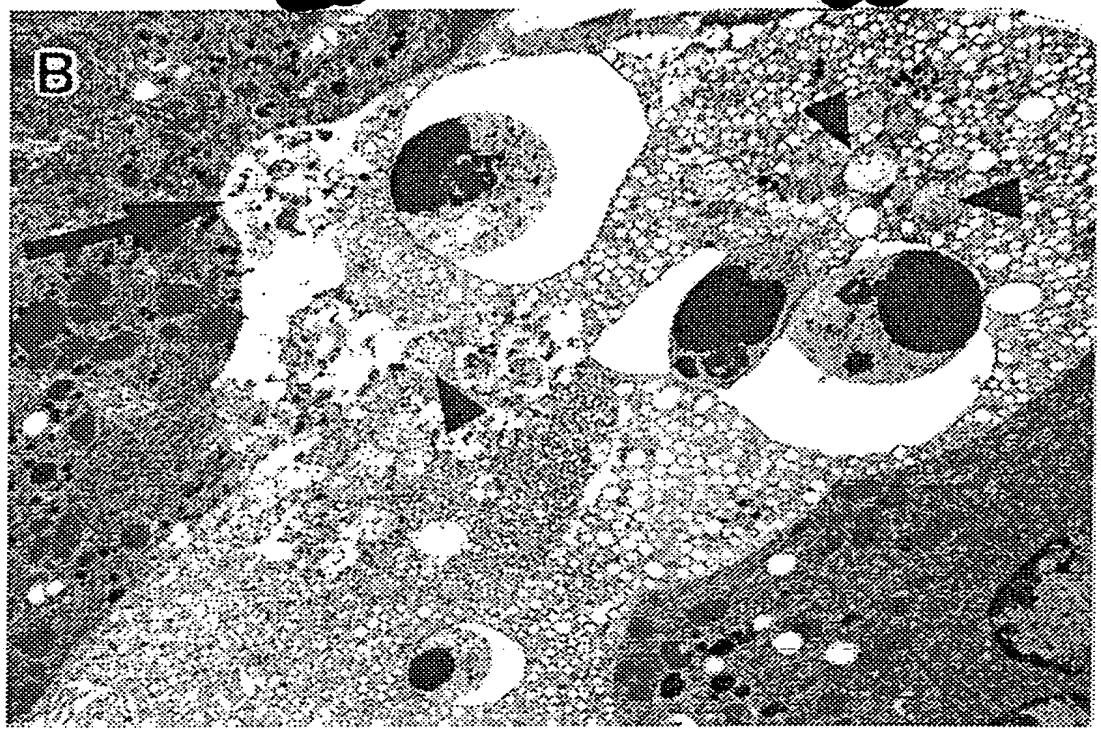

FIGURE 5

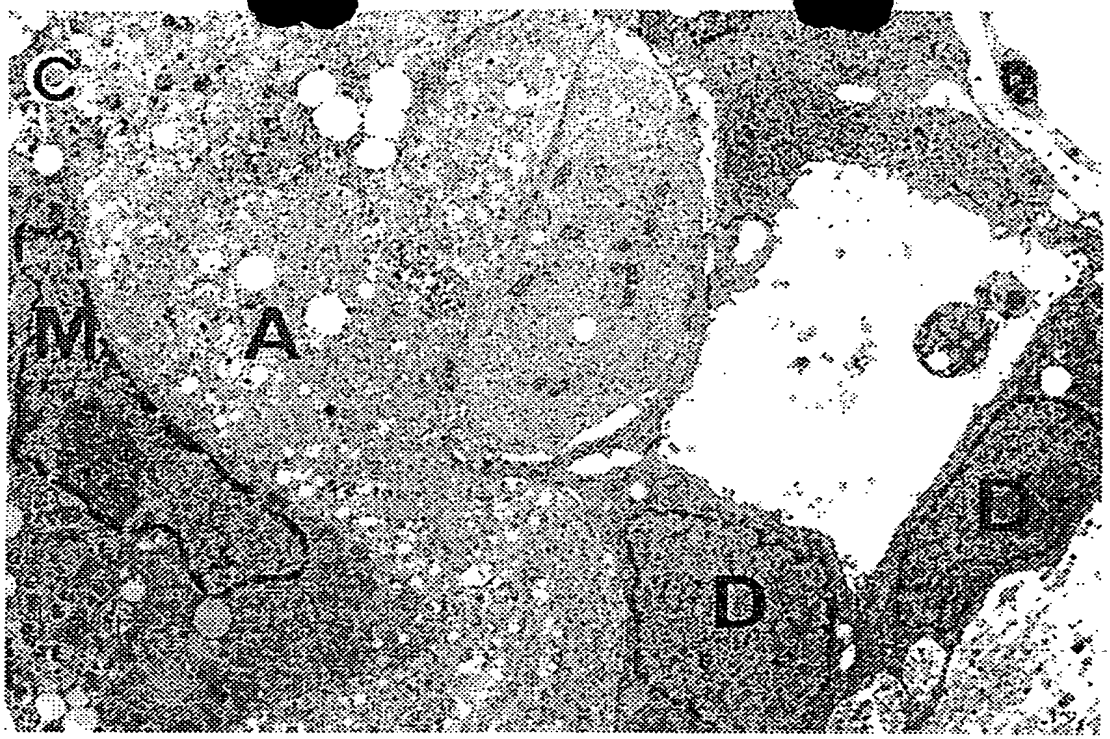

FIGURE 5

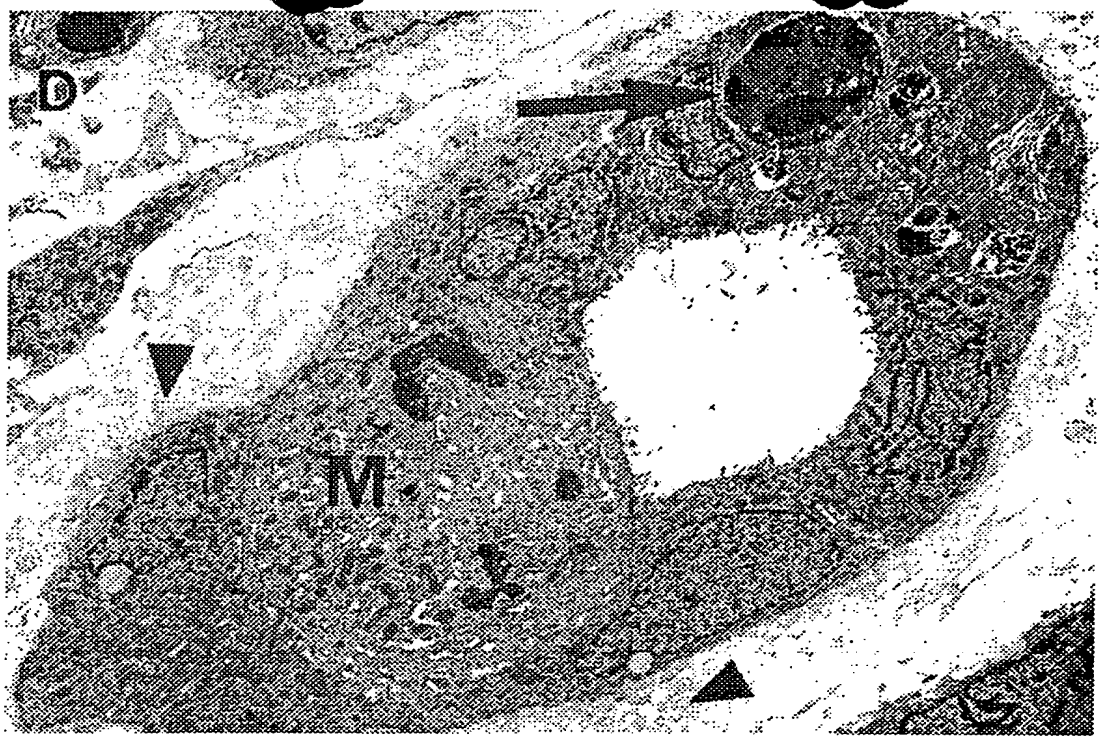

FIGURE 5

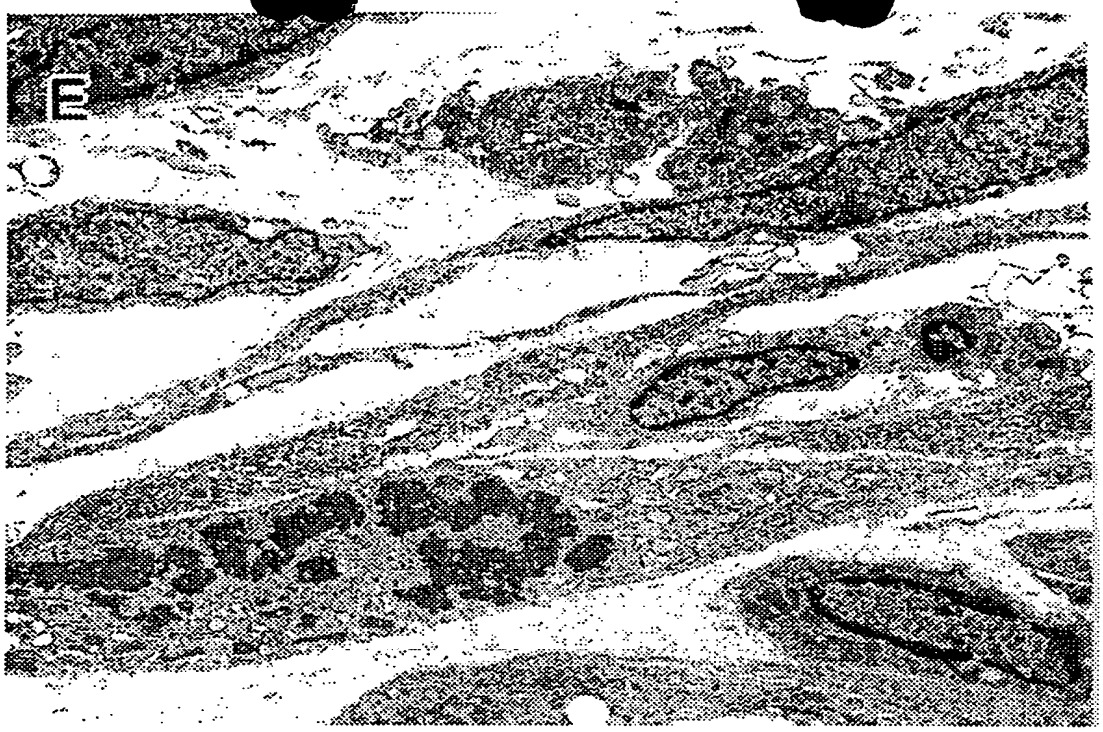

FIGURE 5

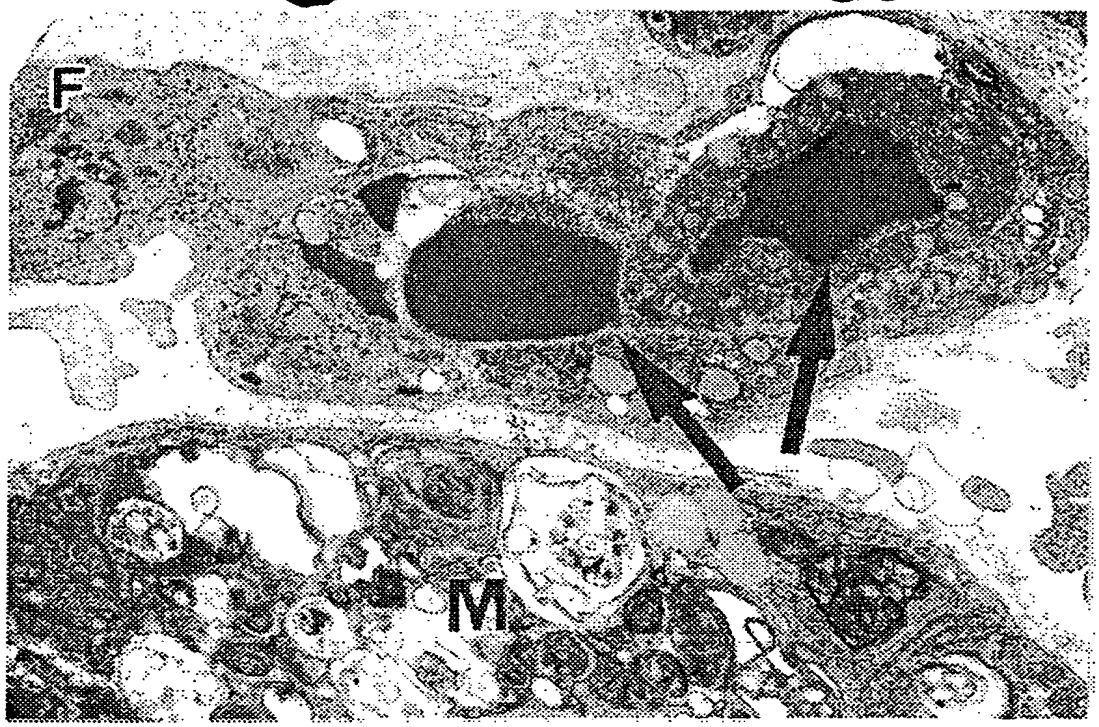

FIGURE 5

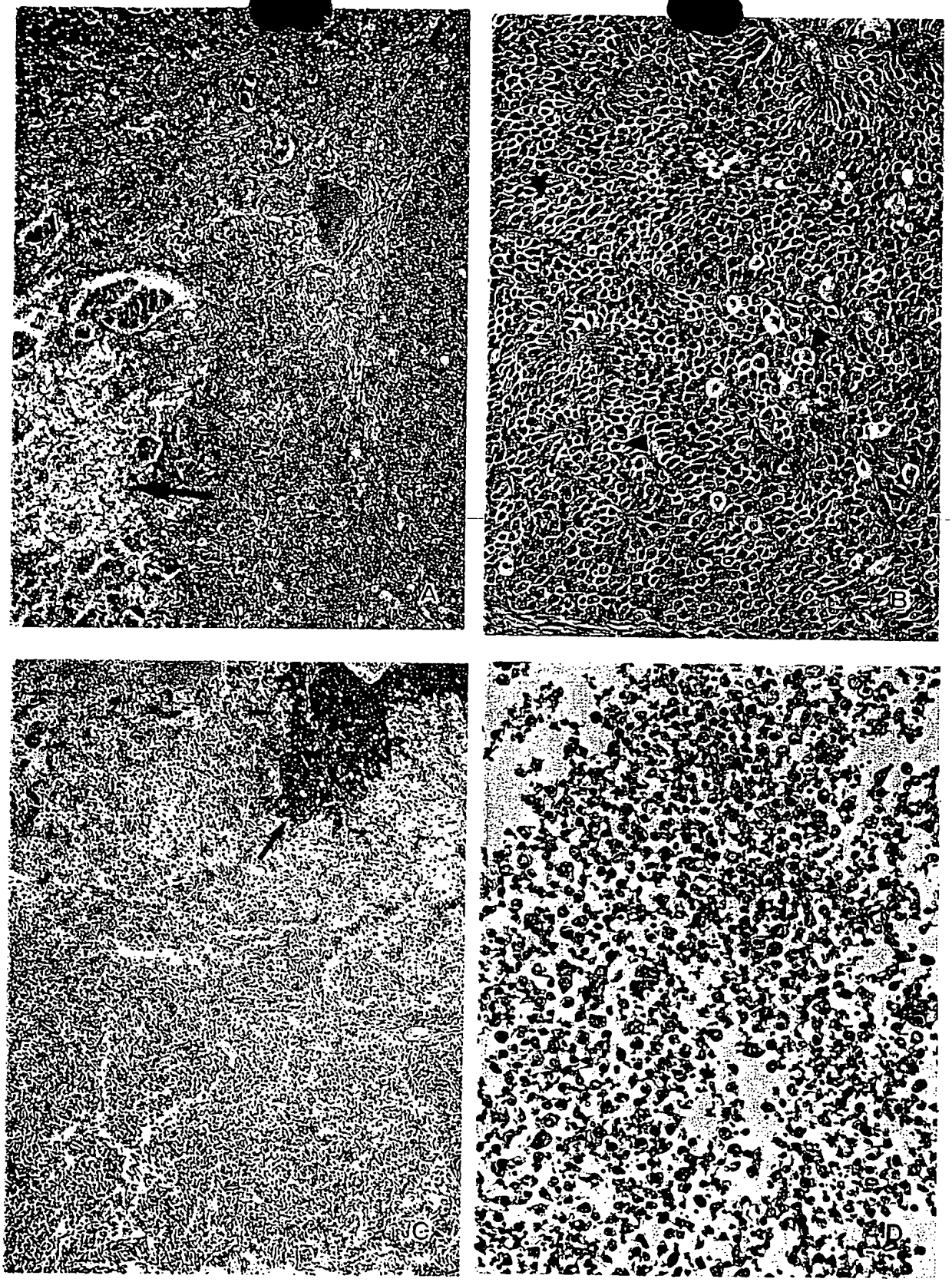

Figure **6**

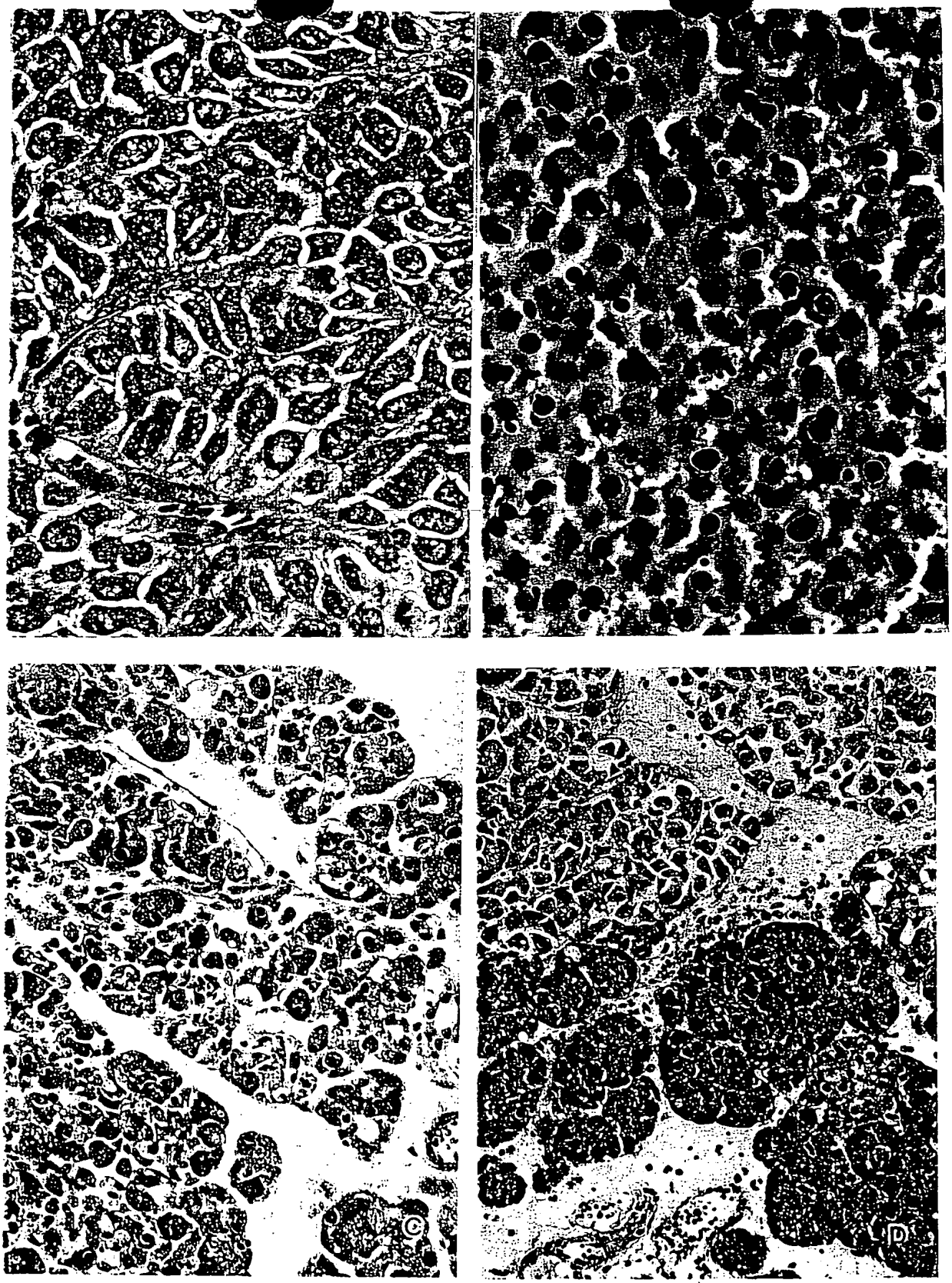

Figure 7

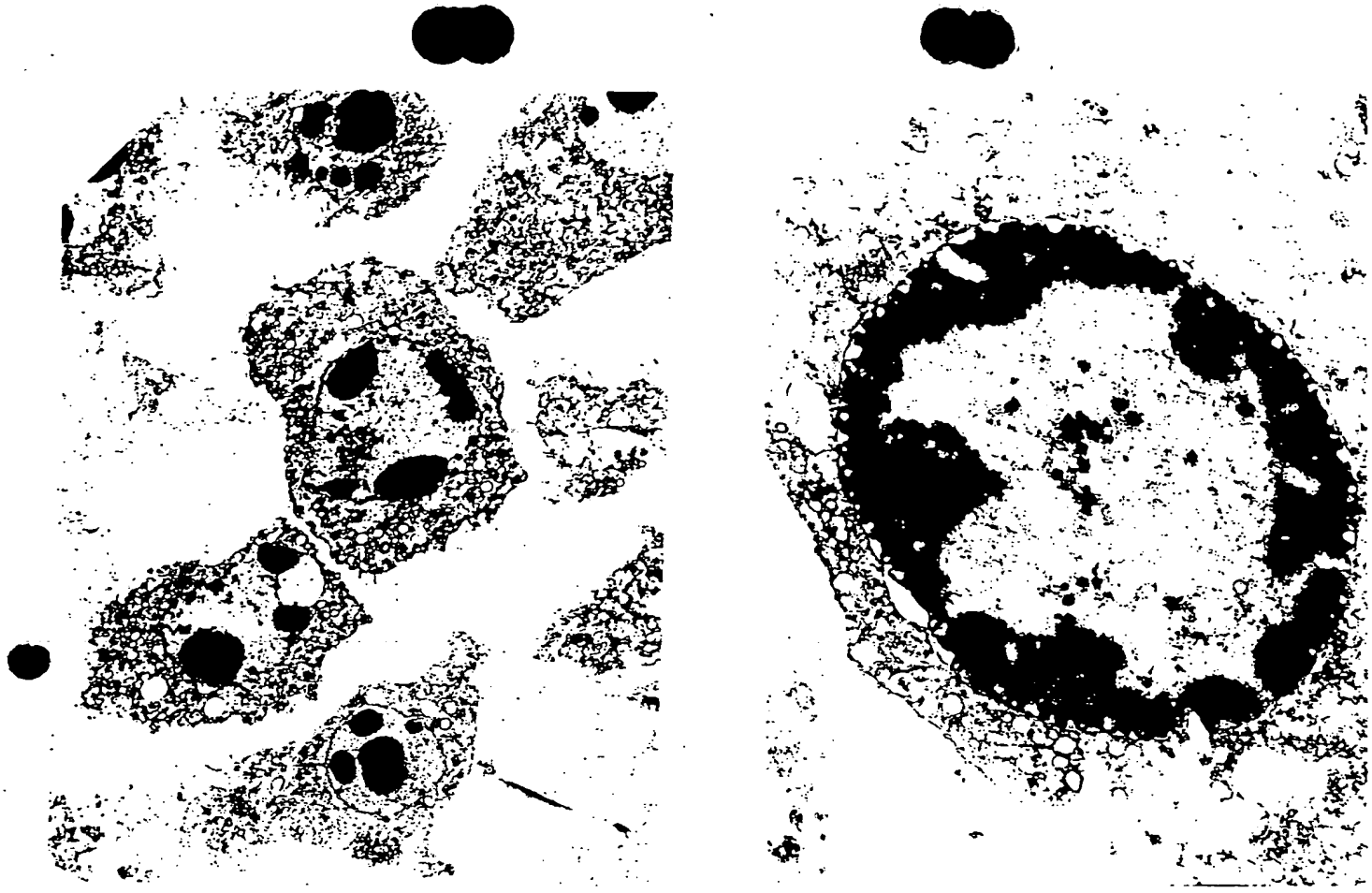

FIGURE 8